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**SIGMA-1 RECEPTOR ACTIVATION PREVENTS INTRACELLULAR CALCIUM
DYSREGULATION IN CORTICAL NEURONS DURING IN VITRO ISCHEMIA***

by

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Running Title: Sigma-1 receptor block of ischemia-induced Ca^{2+} transients

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Number of text pages: 31

Number of tables: 0

Number of figures: 9

Number of references: 40

Abstract: 248 words

Introduction: 747 words

Discussion: 1471 words

Abbreviations: DTG, 1,3-di-*o*-tolyl-guanidine; NMDA, *N*-methyl-D-aspartate; $[\text{Ca}^{2+}]_i$, intracellular calcium concentration; $\Delta[\text{Ca}^{2+}]_i$, change in intracellular calcium concentration; AM, acetoxymethyl ester; (+)-PTZ, (+)-pentazocine; PCP, phencyclidine; BD-1047, *N*-[2-3,4-dichlorophenyl)-ethyl]-*N*-methyl-2-(dimethylamino)ethylamine; MK-801, (+)-5-methyl-10,11-dihydro-5H-dibenzo(a,d)cyclohepten-5,10-imine maleate; PRE-089, 2-(4-Morpholinethyl) 1-phenylcyclohexanecarboxylate hydrochloride; (+)-SKF-10,047, [2*S*-(2 α ,6 α ,11*R**)]-1,2,3,4,5,6-hexahydro-6,11-dimethyl-3-(2-propenyl)-2,6-methano-3-benzazocin-8-ol hydrochloride; PSS, physiological saline solution; RR, ruthenium red; D-AP5, D-2-amino-5-phosphonopentanoic acid.

Section assignment: Neuropharmacology

ABSTRACT

Sigma receptors are putative targets for neuroprotection following ischemia; however, little is known on their mechanism of action. One of the key components in the demise of neurons following ischemic injury is the disruption of intracellular calcium homeostasis. Fluorometric calcium imaging was used to examine the effects of sigma receptor activation on changes in intracellular calcium concentrations ($[Ca^{2+}]_i$) evoked by in vitro ischemia in cultured cortical neurons from embryonic rats. The sigma receptor agonist, DTG, was shown to depress $[Ca^{2+}]_i$ elevations observed in response to ischemia induced by sodium azide and glucose deprivation. Two sigma receptor antagonists, metaphit and BD-1047, were shown to blunt the ability of DTG to inhibit ischemia-evoked increases in $[Ca^{2+}]_i$, revealing that the effects are mediated by activation of sigma receptors and not via the actions of DTG on non-specific targets such as NMDA receptors. DTG inhibition of ischemia-induced increases in $[Ca^{2+}]_i$ was mimicked by the sigma-1 receptor-selective agonists, carbetapentane, (+)-pentazocine and PRE-084, but not by the sigma-2 selective agonist, ibogaine, showing that activation of sigma-1 receptors is responsible for the effects. In contrast, DTG, carbetapentane and ibogaine blocked spontaneous, synchronous calcium transients observed in our preparation at concentrations consistent with sigma receptor mediated effects, indicating that both sigma-1 and sigma-2 receptors regulate events that affect $[Ca^{2+}]_i$ in cortical neurons. Our studies show that activation of sigma receptors can ameliorate $[Ca^{2+}]_i$ dysregulation associated with ischemia in cortical neurons, and thus identify one of the mechanisms by which these receptors may exert their neuroprotective properties.

INTRODUCTION

Sigma receptors are widely distributed in the mammalian brain; and these receptors recognize a diverse array of centrally acting substances including opiates, antipsychotics, antidepressants, phencyclidine (PCP)-related compounds, and neurosteroids (Walker et al., 1990; Bowen, 2000). Thus far, two sigma receptor subtypes have been identified on the basis of their pharmacological profile, with the sigma-1 receptor showing high affinity for the positive isomer of bezomorphans such as (+)-pentazocine and (+)-SKF-10,047, and the sigma-2 receptor having high affinity for ibogaine (Vilner and Bowen, 2000); but only the sigma-1 receptor has been cloned (Hanner et al., 1996). Sigma receptors have been implicated in numerous physiological and pathophysiological processes such as learning and memory (Senda et al., 1996), movement disorders (Matsumoto et al., 1990), and drug addiction (McCracken et al., 1999). These receptors are emerging as therapeutic targets for various diseases such as neuropsychiatric disorders and cancer (Casellas et al., 2004; Hayashi and Su, 2004). Moreover, the observation that several sigma receptor ligands are neuroprotective in both in vivo and in vitro models of ischemia has generated interest in targeting these receptors to enhance neuronal survival following stroke (Takahashi et al., 1996); (Lockhart et al., 1995). Recently, our laboratory has shown that activation of sigma receptors is neuroprotective at delayed time points in a rat model of ischemic stroke (Ajmo et al., 2006).

Dysregulation of intracellular calcium homeostasis greatly contributes to the demise of neurons following an ischemic insult in the central nervous system (Mattson, 2000). Elevation of intracellular calcium disrupts plasma membrane function via activation of calcium-sensitive ion channels (Murai et al., 1997), and triggers biochemical cascades that ultimately promote processes such as proteolysis, lipolysis, and the production of reactive oxygen species (Mattson, 2000). It has been suggested that the neuroprotective properties of sigma ligands depend in part on their ability to depress elevations in intracellular calcium associated with

glutamate receptor-mediated excitotoxicity (Klette et al., 1995; Klette et al., 1997). However, the membrane dysfunction produced by ischemia can stimulate multiple plasma membrane calcium fluxes, including some that are independent of glutamate receptor activation (Tanaka et al., 1997). Sigma receptors have been shown to block both voltage-gated calcium channels and ionotropic glutamate receptors (Zhang and Cuevas, 2002; Monnet et al., 2003). Both of these ion channel types are believed to be involved in the dysregulation of intracellular calcium homeostasis accompanying ischemia, and selective inhibitors of these channels have been shown to provide some degree of neuroprotection (Schurr, 2004). Thus, one of the mechanisms by which sigma receptors may prevent these increases in calcium is via the inhibition of multiple plasma membrane calcium channels. However, the role of sigma receptors in the modulation of ischemia-induced elevations in intracellular calcium has not been unequivocally established because studies on the effects of sigma receptors on calcium homeostasis during neuronal injury have examined intracellular calcium changes in response to direct glutamate application rather than in vitro ischemia models. Given that non-glutamate induced calcium fluxes are also a factor following ischemia, it is important to examine sigma receptor modulation of intracellular calcium using an ischemia model. Determining the role of sigma receptors in preserving calcium homeostasis is the first step toward establishing these receptors as a target for neuroprotection.

The studies of sigma receptor modulation of glutamate evoked changes in intracellular calcium have also resulted in considerable controversy in the literature. There are conflicting reports as to whether sigma receptor ligands exert their effects via actions on sigma receptors (Hayashi et al., 1995; Monnet et al., 2003) or non-specific interaction with other targets, in particular, NMDA receptors (Kume et al., 2002). To some extent, analysis and interpretation of the results has been confounded by limitations in the pharmacological approaches used. For example, sigma receptors and NMDA receptors both bind PCP and related compounds (eg. MK-801) with high affinity (Sircar et al., 1987), and thus, such drugs cannot be used to

discriminate between direct and indirect effects. Also, previous studies have not effectively used specific sigma receptor antagonists to confirm results.

Experiments were undertaken to determine if activation of sigma receptors in cultured cortical neurons modulates elevations in intracellular calcium observed in response to in vitro ischemia. Activation of sigma receptors with specific sigma agonists was shown to depress the peak amplitude of ischemia-induced calcium transients, and this effect was blocked sigma receptor-specific antagonists. Moreover, sigma receptor subtype-selective agonists showed that sigma-1 receptors are responsible for the observed depression of calcium elevations evoked by ischemia, whereas both sigma receptor subtypes regulate spontaneous calcium transients observed in cultured cortical neurons.

METHODS

Preparation

The effects of sigma receptors on ischemia-induced changes in intracellular calcium concentrations were studied in cultured cortical neurons from embryonic (E18) rats. All procedures were done in accordance with the regulations of the University of South Florida Institutional Animal Care and Use Committee. Dams were euthanatized by decapitation, uterus removed, and embryos dissected out and placed in isotonic buffer containing (in mM): 137 NaCl, 5 KCl, 0.2 NaH₂PO₄, 0.2 KH₂PO₄, 5.5 glucose, 6 sucrose (pH 7.4 with NaOH). Cortex were excised and minced, and tissue digested in isotonic buffer containing 0.25% trypsin/EDTA for 10 min at 37°C and added to 3x volume of high glucose culture media (Dulbecco's Modified Eagle Media; Invitrogen, Inc., Carlsbad, CA), 10% (v/v) fetal calf serum, 100 U/ml penicillin and 0.1 mg/ml streptomycin. Cells were counted on a hemocytometer, plated (0.5×10^6 cells) on 18 mm coverslips coated with poly-L-lysine, and incubated at 37°C under a 95% air, 5% CO₂ atmosphere. After 24 hr the media was replaced with Neurobasal (Invitrogen) medium

supplemented with B27 (Invitrogen) and 0.5 mM L-glutamine to limit astrocyte proliferation in the cultures. Cells were used after 10-17 days in culture, unless otherwise indicated.

Microfluorometric measurements

Intracellular free-calcium was measured using the calcium sensitive dye, fura-2 as previously described (DeHaven and Cuevas, 2004). Cells plated on coverslips were incubated for 1 hour at room temperature in physiological saline solution (PSS) consisting of (in mM): 140 NaCl, 3 KCl, 2.5 CaCl₂, 1.2 MgCl₂, 7.7 glucose and 10 HEPES (pH to 7.2 with NaOH), which also contained 1 μ M of the membrane permeable ester form of fura-2, acetoxymethylester (fura-2 AM) and 0.1 % dimethyl sulfoxide (DMSO). The coverslips were then washed in PSS (fura-2-AM free) prior to the experiments being carried out. All solutions were applied via a rapid application system identical to that previously described (Cuevas and Berg, 1998).

A DG-4 high-speed wavelength switcher (Sutter Instruments Co., Novato, CA) was used to apply alternating excitation light, and fluorescent emission was captured using a Sensicam digital CCD camera (Cooke Corporation, Auburn Hills, MI) and recorded with Slidebook 3.0 software (Intelligent Imaging Innovations, Denver, CO). Changes in $[Ca^{2+}]_i$ were calculated using the Slidebook 3 software (Intelligent Imaging Innovations, Denver, CO) from the intensity of the emitted fluorescence following excitation with 340 and 380 nm light, respectively, using the Grynkiewicz equation:

$$[Ca^{2+}]_i = K_d Q (R - R_{min}) / (R_{max} - R)$$

where R represents the fluorescence intensity ratio (F_{340}/F_{380}) as determined during experiments, Q is the ratio of F_{min} to F_{max} at 380 nm, and K_d is the Ca^{2+} dissociation constant for fura-2 (225 μ M). The system was calibrated using a Fura-2 Calcium Imaging Calibration Kit (Molecular Probes; Eugene, OR) and values for F_{min}/F_{max} , R_{min} , and R_{max} were determined to be 23.04, 0.2, and 8.4, respectively.

In vitro ischemia.

In vitro ischemia was achieved using the sodium azide/glucose deprivation model. This model for ischemic neuronal injury has been used effectively in numerous studies to mimic in vivo stroke in an in vitro environment, and has been shown to elicit electrophysiological and neurochemical changes that are qualitatively identical to the oxygen/glucose deprivation model of ischemia (Murai et al., 1997) (Finley et al., 2004). The major advantage of the sodium azide/glucose deprivation model over the oxygen/glucose deprivation is that it elicits neurochemical responses that are significantly more rapid and robust (Finley et al., 2004), thus facilitating the recording of changes in $[Ca^{2+}]_i$.

Data analysis

Analyses of these data were conducted using the SigmaPlot 2000 program (SPSS Science, Chicago, IL). Data points represent means $\pm$ standard error of the mean (SEM). Statistical difference was determined using paired *t*-test for within-group experiments and unpaired *t*-test for between group experiments. For multiple group comparison an ANOVA was used followed by post-hoc analysis with a Dunn's test. Differences were considered significant if $p < 0.05$.

Solutions and reagents

The control bath solution for all experiments was a physiological saline solution (PSS) containing (in mM) 140 NaCl, 1.2 MgCl₂, 3 KCl, 2.5 CaCl₂, 7.7 glucose and 10 HEPES, pH to 7.2 with NaOH. For experiments with no extracellular calcium, CaCl₂ was excluded from the PSS. All drugs were applied in this solution unless otherwise noted. In vitro ischemia was induced by addition of the cytochrome oxidase inhibitor, NaN₃ (4 mM), and removal of glucose from the PSS. For experiments in which multiple ischemic episodes were induced in a single

cell, the order of drug application was alternatively reversed to compensate for any effects due to rundown, desensitization or ischemic preconditioning. For experiments with metaphit, cells were preincubated in 50 μ M metaphit during the last 15 min of fura-2 loading and immediately prior to experiments being conducted. The metaphit was washed off for 5 min in the bath using PSS.

All chemicals used in this investigation were of analytic grade. The following drugs were used: ruthenium red, tetrodotoxin (TTX), DTG, ibogaine and metaphit (Sigma-Aldrich, St. Louis, MO); D-AP5, BD-1047, carbetapentane, and PRE-084 (Tocris Bioscience, Ellisville, MO); ryanodine and thapsigargin (Alomone Labs, Jerusalem, Israel); and fura-2-AM (Molecular Probes, Eugene, OR).

RESULTS

Experiments were conducted to characterize the changes in intracellular calcium evoked by the sodium-azide/glucose deprivation model of in vitro ischemia. Figure 1A shows representative traces of change in intracellular Ca^{2+} as a function of time evoked by rapid induction of chemical ischemia in two cortical neurons that had remained in culture for 3 or 14 days, respectively. Following 3 days in culture, chemical ischemia elicited small, slow rising elevations in $[\text{Ca}^{2+}]_i$ in the neurons, whereas after 14 days in culture, ischemia evoked rapid increases in $[\text{Ca}^{2+}]_i$. The increases in $[\text{Ca}^{2+}]_i$ observed at later time points (7-21 days in culture) in response to ischemia were transient, and $[\text{Ca}^{2+}]_i$ returned to control levels in >80% of the cells tested following washout with control PSS (n > 1000). A plot of mean peak change in $[\text{Ca}^{2+}]_i$ shows that the response to chemical ischemia increased significantly from 3 to 14 days in culture, and the elevations in $[\text{Ca}^{2+}]_i$ diminished when the neurons were in culture for over 14 days (Figure 1B).

Previous studies have shown that chemical ischemia promotes the release of excitatory neurotransmitters which may elicit these elevations in $[\text{Ca}^{2+}]_i$ (Djali and Dawson, 2001).

Therefore, experiments were conducted to determine if inhibition of voltage-activated Na^+ channels, and consequently neurotransmission, with TTX (200 nM) abolished the elevations in $[\text{Ca}^{2+}]_i$ induced by chemical ischemia. Figure 1C shows representative $[\text{Ca}^{2+}]_i$ traces of responses evoked by ischemia in the absence and presence of TTX. Inhibition of synaptic transmission with TTX depressed ischemia-evoked increases in $[\text{Ca}^{2+}]_i$ relative to control. A plot of maximal increase in $[\text{Ca}^{2+}]_i$ in the absence and presence of TTX is shown in Figure 1D. The ischemia-evoked increase in $[\text{Ca}^{2+}]_i$ was decreased in a significant manner by $76 \pm 4\%$ in the presence of TTX.

Further experiments were conducted to resolve the source of calcium mediating the elevations in $[\text{Ca}^{2+}]_i$ observed in response to chemical ischemia. To determine if extracellular calcium contributed to the increase in $[\text{Ca}^{2+}]_i$, ischemic conditions were induced in the absence and presence of extracellular calcium. Under both conditions, elevations in $[\text{Ca}^{2+}]_i$ were noted (Figure 2A), but in the absence of extracellular calcium, the peak increases in $[\text{Ca}^{2+}]_i$ were significantly less than those observed in control experiments (Figure 2B). Thus, a component of the ischemia-induced increase in $[\text{Ca}^{2+}]_i$ depends on the presence of extracellular calcium. To gain further insight into the ion channel type mediating the influx of calcium following ischemia we used the NMDA receptor-selective antagonist, D-AP5. D-AP5 (100 μM) had no effect on basal $[\text{Ca}^{2+}]_i$, but depressed peak $[\text{Ca}^{2+}]_i$ following ischemia (Figure 2C). However, the decrease in ischemia-induced increase in $[\text{Ca}^{2+}]_i$ observed in the presence of D-AP5, was less than that produced by the non-selective calcium channel blocker La^{3+} (10 μM ; Figure 2D). Our data show that that NMDA receptors are responsible for $\sim 35\%$ of the calcium influx evoked in this ischemia model. Due to the fact that La^{3+} blocks $\sim 90\%$ of the elevation in $[\text{Ca}^{2+}]_i$, and that the concentration of La^{3+} used here (10 μM) blocks voltage-gated calcium channels, NMDA receptors, and kainate receptors, but not AMPA receptors (Huettner et al., 1998), AMPA

receptors are likely to be minor contributors to the elevations in $[Ca^{2+}]_i$ evoked by this chemical ischemia model.

Given that elimination of extracellular calcium did not abolish the increase in $[Ca^{2+}]_i$, we investigated the role of calcium release from intracellular stores in the response to ischemia. Ryanodine (10 μ M) was used to selectively inhibit release from caffeine/ryanodine-sensitive calcium stores; whereas, the sarcoplasmic/endoplasmic reticulum Ca^{2+} -ATPase inhibitor, thapsigargin (10 μ M), was used to deplete both ryanodine- and IP_3 -sensitive stores. Ruthenium red was used to block any calcium efflux from the mitochondria via reversal of the calcium uniporter. Ischemia-increases in $[Ca^{2+}]_i$ were observed in control, ryanodine, thapsigargin, and ruthenium red experiments (Figure 2E). However, preincubation in thapsigargin decreased the peak elevation in $[Ca^{2+}]_i$, whereas ryanodine did not significantly alter the effects of ischemia on $[Ca^{2+}]_i$ (Figure 2F). Ruthenium red also failed to block ischemia-induced increases in $[Ca^{2+}]_i$, and was associated with an increase in $[Ca^{2+}]_i$ (Figure 2F). This increase is likely due to ruthenium red preventing calcium uptake (buffering) by the mitochondria, which is known to occur in neurons in response to cytosolic elevations in calcium (Wang and Thayer, 2002). Taken together these data suggest that elevations of $[Ca^{2+}]_i$ observed in response to chemical ischemia are in part due to calcium release from IP_3 -sensitive stores, but do not appear to involve liberation of calcium from ryanodine sensitive stores or the mitochondria.

Experiments were carried out to determine if stimulation of sigma receptors modulates the changes in $[Ca^{2+}]_i$ evoked by in vitro ischemia. Figure 3A shows representative traces of $[Ca^{2+}]_i$ recorded from a single neuron in response to ischemia in the absence (Control) and presence of 50 μ M DTG (DTG). The elevation in $[Ca^{2+}]_i$ evoked by ischemia was abolished when the sigma receptor ligand was co-applied. A bar graph of ischemia-induced mean peak increase in $[Ca^{2+}]_i$ observed in 13 neurons in the absence and presence of 50 μ M DTG is shown

in Figure 3B, and demonstrates that DTG decreases the rise in $[Ca^{2+}]_i$ by ~70%. This effect of DTG was statistically significant and was reversible upon washout of the sigma agonist (data not shown). Similar results were obtained for DTG inhibition of net change in $[Ca^{2+}]_i$ (area under the curve) and $[Ca^{2+}]_i$ amplitude at the end of the ischemic episode (data not shown).

The magnitude of the DTG-mediated block of elevations in $[Ca^{2+}]_i$ suggests that sigma receptors are modulating calcium elevations evoked by the various components that contribute to the ischemia-induced dysregulation of $[Ca^{2+}]_i$. To test this possibility, DTG (100 μ M) was applied under various conditions targeting specific components of the increase in $[Ca^{2+}]_i$. DTG significantly decrease ischemia-evoked elevations in $[Ca^{2+}]_i$ when intracellular calcium stores were depleted by thapsigargin preincubation (10 μ M, 1 hr, 23° C) or when the mitochondrial calcium uniporter was blocked with ruthenium red (20 μ M) (Figure 3C). Similarly, DTG blocked calcium elevations evoked by ischemia following NMDA receptor inhibition with D-AP5 (100 μ M) or when calcium influx was blocked by removal of extracellular calcium or application of the pan-specific calcium channel blocked, La^{3+} (10 μ M) (Figure 3C). However, DTG failed to block the residual calcium elevation evoked by ischemia in the presence of TTX. Thus, sigma receptor activation blocks calcium elevations associated with both calcium entry through the plasma membrane and calcium release from intracellular stores. However, the effects of sigma receptor activation appear to be limited to elevations in $[Ca^{2+}]_i$ associated with synaptic transmission in this model of ischemia.

To confirm that the effects of DTG on $[Ca^{2+}]_i$ are mediated via the activation of sigma receptors, the sigma receptor antagonist, metaphit, was used in a series of experiments. Cells were exposed to ischemia in the absence and presence of 50 μ M DTG, with or without preincubation in metaphit (50 μ M). Whereas DTG depressed the ischemia induced elevation in $[Ca^{2+}]_i$ in control cells, the responses observed in cells pretreated with metaphit were comparable in the absence and presence of DTG (Figure 4A). Moreover, both responses ($\pm$

DTG) observed in metaphit pretreated cells were larger than the control response (no metaphit pretreatment). In similar experiments, cells not exposed to metaphit responded to DTG with a decrease in ischemia-induced elevations in $[Ca^{2+}]_i$ from a control value of 291 ± 47 nM to 47 ± 9 nM in the presence of the sigma agonist (Figure 4B). Cells preincubated in metaphit displayed a more robust increase in $[Ca^{2+}]_i$ during ischemia (495 ± 68 nM) relative to control cells (Figure 4B). Furthermore, neurons pretreated with metaphit continued to exhibit pronounced elevations in $[Ca^{2+}]_i$ in the presence of DTG (237 ± 39 nM). These increases in $[Ca^{2+}]_i$ were significantly greater ($p < 0.01$) than those observed in control neurons exposed to DTG and were comparable to those seen in control neurons not exposed to DTG. To confirm that the difference observed in responses to DTG and DTG following metaphit preincubation were not the result of metaphit augmentation of the $[Ca^{2+}]_i$ responses, the responses were normalized to the mean of their respective controls. Figure 4C shows a bar graph of the relative change in $[Ca^{2+}]_i$ observed in the presence of DTG in control neurons (DTG) and neurons preincubated in metaphit (MET + DTG). Whereas DTG decreased the elevation in $[Ca^{2+}]_i$ evoked by ischemia in control cells by $83 \pm 3\%$, the sigma receptor agonist only reduced the response by $52 \pm 8\%$ in cells preincubated in the irreversible sigma receptor antagonist.

A second sigma receptor-selective antagonist, BD-1047 (Matsumoto et al., 1995), was used to further support that DTG was acting via the stimulation of sigma receptors. Figure 5A shows intracellular calcium traces obtained from three neurons in response to chemical ischemia in the absence (Control) and presence of 10 μ M DTG (DTG) or 10 μ M DTG following a 5 min preincubation in 10 μ M BD-1047 (DTG + BD-1047). While DTG reduced the ischemia-elicited elevations in $[Ca^{2+}]_i$, application of BD-1047 diminished the effectiveness of DTG. In similar experiments, the effects of DTG on ischemia-evoked calcium transients were blocked by 1 μ M and 10 μ M BD-1047 in a concentration-dependent and statistically significant manner

(Figure 5B). These two concentrations of BD-1047 reduced the effects of DTG by 15% and 55%, respectively.

DTG and metaphit are pan-selective sigma ligands, acting on both sigma-1 and sigma-2 receptors, and the concentrations of BD-1047 used here cannot definitively discriminate between the receptor subtypes. Therefore, experiments were conducted using sigma receptor subtype-selective agonists to determine the specific sigma receptor subtype(s) responsible for the depression of ischemia induced increases in $[Ca^{2+}]_i$. Figure 6A shows representative traces of $[Ca^{2+}]_i$ recorded from three neurons in the absence (Control) and presence of the sigma-1 selective agonist, carbetapentane, at the indicated concentrations. Carbetapentane reduced the effect of ischemia on $[Ca^{2+}]_i$ in a concentration dependent manner, and this effect of carbetapentane was reversible upon washout of drug (data not shown). Figure 6B shows a plot of the relative ischemia-induced increases in $[Ca^{2+}]_i$ as a function of carbetapentane concentration. A fit of the data using a Langmuir-Hill equation indicated that the sigma-1 selective ligand inhibits the effects of ischemia on $[Ca^{2+}]_i$ with a half-maximal concentration of 13.3 μ M and with a Hill Coefficient of 0.8.

Additional experiments were conducted to determine if sigma-2 receptors contribute to the DTG-mediated inhibition of ischemia-induced increases in $[Ca^{2+}]_i$. For these experiments the sigma-2 receptor-selective agonist, ibogaine, was used. Figure 7A shows representative traces of $[Ca^{2+}]_i$ recorded in response to ischemia in the absence and presence of 100 μ M ibogaine. Unlike carbetapentane, ibogaine failed to inhibit the ischemia-induced elevations in $[Ca^{2+}]_i$. In similar experiments, ibogaine at a concentration range of 1-100 μ M, which has been shown to block sigma-2 mediated events (Zhang and Cuevas, 2002), failed to inhibit the effects of ischemia on $[Ca^{2+}]_i$ (Figure 7B). This observation suggests that the sigma-1 receptor is primarily responsible for the depression of ischemia-induced increase in $[Ca^{2+}]_i$ mediated by DTG.

To confirm that sigma-1 activation attenuates ischemia-induced increase in $[Ca^{2+}]_i$ mediated by DTG and carbetapentane, neurons were treated with the sigma-1 selective agonists (+)-pentazocine and PRE-084. Figure 8A shows representative traces of $[Ca^{2+}]_i$ recorded during ischemia from 3 neurons in the absence (Control) and presence of (+)-pentazocine at the indicated concentrations. Peak elevations in $[Ca^{2+}]_i$ were significantly depressed by (+)-pentazocine in a concentration dependent manner. Application of 10 μ M (+)-pentazocine decreased the ischemia-induced elevations in $[Ca^{2+}]_i$ by $37 \pm 5\%$, whereas 100 μ M (+)-pentazocine depressed the change in $[Ca^{2+}]_i$ by $49 \pm 4\%$ (Figure 8B). Responses to ischemia were also blocked by application of PRE-084 (Figure 8C). Both 10 μ M and 100 μ M PRE-084 decreased elevations in $[Ca^{2+}]_i$ in a statistically significant manner. These decreases were $26 \pm 4\%$ and $58 \pm 1\%$, respectively (Figure 8D).

Spontaneous elevations in $[Ca^{2+}]_i$ were frequently observed in our experiments (see Figure 8C, Control trace). These elevations in $[Ca^{2+}]_i$ nearly always occurred in multiple neurons in the same visual field in a synchronized manner (data not shown). While our data demonstrate that activation of sigma receptors decreased the ischemia-induced elevations of $[Ca^{2+}]_i$, further experiments were conducted to determine if spontaneous increases in $[Ca^{2+}]_i$ were also modulated by sigma receptors. Figure 9A shows traces of spontaneous activity recorded from a single cortical neuron in the absence (Control) and presence of 100 μ M DTG (*i.*) and following washout of drug (Wash). DTG was found to reversibly block spontaneous increases in $[Ca^{2+}]_i$ in a statistically significant manner (Figure 9B). To identify the subtype of sigma receptor involved in the modulation of spontaneous calcium transients, the sigma-2-selective agonist, ibogaine, was used. Traces of spontaneous activity recorded from a single cortical neuron in the absence (Control, Wash) and presence of 50 μ M ibogaine (IBO) are shown in Figure 9A, *ii.* In identical experiments, bath application of the sigma-2 agonist significantly decreased the number of spontaneous calcium events (Figure 9C). Activation of

sigma-1 receptors with carbetapentane (100 μ M) also affected spontaneous activity (Figure 9A, *iii*), resulting in a significant decrease in the number spontaneous of Ca^{2+} transients observed in the cells (Figure 9D).

DISCUSSION

The major finding reported here is that activation of sigma-1 receptors depresses ischemia-induced dysregulation of intracellular calcium in cultured cortical neurons by affecting multiple pathways that contributing to the rise in intracellular calcium concentrations. This effect of sigma receptors is primarily due to the modulation of intracellular calcium increases resulting from the stimulation of synaptic transmission elicited by ischemia. Pharmacological studies using sigma-selective agonists and antagonists clearly show that the effects of the sigma ligands are mediated by actions on sigma receptors and are not the result of modulation of NMDA receptors by the drugs. Our data also indicate that tonic activation of sigma receptors or activation of sigma receptors upon induction of ischemia via an endogenous mechanism diminishes ischemia-induced elevations of intracellular calcium. Finally, our findings also demonstrate that while sigma-2 receptors do not appreciably influence ischemia-induced changes in $[\text{Ca}^{2+}]_i$, these receptors can decrease spontaneous calcium transients observed in cultured cortical neurons.

Previous studies have shown that the sigma ligands (+)SKF10047 (10 μ M) and haloperidol (10 μ M), but not carbetapentane (100 μ M) or DTG (100 μ M), inhibit calcium elevations evoked by glutamate application (Kume et al., 2002; Klette et al., 1995). Our studies show that DTG effectively blocks ischemia-induced elevations in $[\text{Ca}^{2+}]_i$ at concentrations that have little or no effect on the rise in $[\text{Ca}^{2+}]_i$ elicited by direct glutamate application (Klette et al., 1995). In contrast, similar concentrations DTG (65 μ M) have been shown to stimulate sigma receptor modulation of electrical activity in frog pituitary melanotrope cells (Soriani et al., 1998).

Low concentrations of carbetapentane (10 μ M) were shown to inhibit ~50% of the peak ischemia-induced increases in $[Ca^{2+}]_i$, whereas 10-fold higher concentrations of this sigma receptor agonist failed to block glutamate-induced increases in $[Ca^{2+}]_i$ (Kume et al., 2002). Moreover, DTG was shown to block a major component of the ischemia-induced increase in $[Ca^{2+}]_i$ that is insensitive to the NMDA receptor antagonist, D-AP5. DTG even blocked a component of the ischemia-induced calcium elevation which was insensitive to La^{3+} (10 μ M), which blocks voltage-gated calcium channels, NMDA receptors, kainate receptors and other Ca^{2+} channel types at this concentration (Huettner et al., 1998). Taken together, these data indicate that the effects of sigma ligands on ischemia-induced increases in $[Ca^{2+}]_i$ cannot be exclusively explained by the actions of these drugs on metabotropic and ionotropic glutamate receptors alone, and are the result of their action on sigma receptors and consequently, on effector targets of sigma receptors.

The role of sigma receptors in the depression of ischemia-elicited increases in $[Ca^{2+}]_i$ is further supported by experiments using the sigma receptor selective antagonists metaphit and BD-1047. Our laboratory has previously shown that metaphit is an irreversible inhibitor of sigma receptors, and that preincubation of neurons in metaphit inhibits sigma-1 and sigma-2 receptor block of K^+ and Ca^{2+} channels, respectively (Zhang and Cuevas, 2002; Zhang and Cuevas, 2005). It is important to note that while metaphit, a PCP analog, can attenuate phencyclidine-induced antagonism of NMDA responses, it does not have any direct effects on NMDA mediated responses even at concentrations significantly greater than those used here (Wang and Lee, 1991). BD-1047 was also shown to block DTG-mediated inhibition of ischemia related dysregulation of $[Ca^{2+}]_i$ at concentrations selective for sigma receptors (Matsumoto et al., 1995). BD-1047 has also been used previously to show that sigma receptors mediate DTG-evoked hypothermia and the effects of cocaine on conditioned place preference (Rawls et al., 2002; Romieu et al., 2004).

The observation that the sigma-1 selective agonists (+)-pentazocine, PRE-084, and carbetapentane, but not the sigma-2-selective agonist, ibogaine, mimicked the effects of DTG on ischemia-induced elevations in $[Ca^{2+}]_i$, indicates that sigma-1 receptors are responsible for the observed effects. Studies have shown that the affinity of sigma-1 receptors for carbetapentane is >30-fold greater than that of sigma-2 receptors, whereas the affinity of sigma-2 receptors for ibogaine is >40-fold greater than that of sigma-1 receptors (Vilner and Bowen, 2000). The calculated IC_{50} for carbetapentane inhibition of ischemia-evoked increases in $[Ca^{2+}]_i$ (13 μ M) is similar to values reported for carbetapentane inhibition of epileptiform activity via sigma receptors in rat hippocampal slices (38 μ M) (Thurgur and Church, 1998). Furthermore, the affinity of sigma-1 receptors for (+)-pentazocine is ~2000-fold greater than for ibogaine, whereas the affinity of sigma-2 receptors for ibogaine is ~6-fold higher than for (+)-pentazocine (Vilner and Bowen, 2000). Here we show that 10 μ M (+) pentazocine blocked ~40% of ischemia-evoked increases in $[Ca^{2+}]_i$, which is consistent with the IC_{50} for (+) pentazocine inhibition of delayed outwardly rectifying K^+ channels (37 μ M) and voltage-gated K^+ channels (42 μ M) via sigma-1 receptors in frog pituitary melanotrophs and rat intracardiac neurons, respectively (Soriani et al., 1998; Zhang and Cuevas, 2005). In contrast, in rat intracardiac neurons sigma-2 receptors inhibit voltage-gated Ca^{2+} , and the IC_{50} reported for ibogaine is 31 μ M (Zhang and Cuevas, 2002). Even at 3-fold higher concentrations, ibogaine failed to affect the ischemia-induced elevations in $[Ca^{2+}]_i$. Thus, the attenuation of elevations in $[Ca^{2+}]_i$ is primarily the result of sigma ligands acting on sigma-1 receptors in cortical neurons.

The mechanism by which sigma-1 receptors modulate ischemia-induced elevations in $[Ca^{2+}]_i$ remains to be fully elucidated. However, several factors are likely involved due to the complex nature of the responses observed in our ischemia model. The dependence of the peak amplitude of ischemia-induced elevations in $[Ca^{2+}]_i$ on the number of days the neurons are in culture coincides with the development of synapses in this preparation, suggesting that the

phenomenon involves synaptic transmission. Also consistent with this hypothesis is the fact that either application of TTX or La^{3+} , or removal of extracellular calcium, significantly depressed $[\text{Ca}^{2+}]_i$ responses. One possibility is that sigma-1 receptor activation is primarily exerting its effects by decreasing glutamate release evoked by ischemia. This would explain the observation that DTG fails to inhibit the small elevations in $[\text{Ca}^{2+}]_i$ produced by ischemia when synaptic transmission is blocked with TTX. Studies have shown that DTG can decrease glutamate release evoked by oxygen and glucose deprivation from hippocampal brain slices (Lobner and Lipton, 1990). Alternatively, sigma-1 receptors may be modulating postsynaptic receptors, and inhibiting neurotransmission. It has been suggested that sigma receptors may block calcium responses evoked by direct activation of both ionotropic and metabotropic glutamate receptors (Klette et al., 1997). The fact that sigma-1 receptor activation can eliminate the elevations in $[\text{Ca}^{2+}]_i$ suggests that they are blocking calcium entry through the plasma membrane and calcium release from intracellular stores, consistent with inhibition of both glutamate receptor subtypes.

An interesting observation reported here is that inhibition of sigma receptors resulted in elevations in basal $[\text{Ca}^{2+}]_i$ and potentiated the increases in $[\text{Ca}^{2+}]_i$ evoked by ischemia. Thus, sigma receptors appear to be involved in calcium homeostasis in cortical neurons under control conditions. Previous studies have shown that sigma receptors can modulate various plasma membrane calcium channels, including voltage-gated Ca^{2+} channels and NMDA receptors (Hayashi et al., 1995; Zhang and Cuevas, 2002), and can regulate phosphatidylinositol turnover (Hayashi et al., 2000). These findings have led to the theory that one of the critical cellular functions of sigma receptors is the regulation of intracellular calcium levels (Hayashi et al., 2000). The observations reported here lend further support to this theory. Moreover, it appears that changes in intracellular calcium are modulated by both sigma-1 and sigma-2 receptors. While sigma-1 receptors affect the ischemia-induced changes in $[\text{Ca}^{2+}]_i$, both sigma receptor subtypes can depress spontaneous calcium transients observed in cultured cortical

neurons. Low concentrations of DTG and ibogaine depressed the genesis of these calcium transients, consistent with a sigma-2 mediated effect. However, the fact that carbetapentane also inhibited these spontaneous Ca^{2+} transients suggests that sigma-1 receptors may also regulate this phenomenon. It remains to be determined if sigma-1 and sigma-2 receptors affect this spontaneous activity via actions on identical targets (e.g. ion channel, calcium store, etc.). These spontaneous increases in $[\text{Ca}^{2+}]_i$ have a frequency that is similar to bursts of spontaneous action potentials observed in our preparation (data not shown), but the exact source and triggering mechanism for these calcium transients remains to be determined. Previous studies have reported these synchronous calcium transients, and they appear to be correlated with bursts of electrical activity, axon outgrowth and synaptic development (Robinson et al., 1993; Tang et al., 2003).

In conclusion, our studies show that sigma-1 receptors mediate the depression of ischemia-induced elevations in $[\text{Ca}^{2+}]_i$. Findings reported here clearly establish that sigma ligands can affect cellular function during ischemia and the concomitant excitotoxicity by acting on sigma receptors, rather than through non-specific effects on other molecular targets. Given that intracellular calcium dysregulation greatly contributes to the demise of cortical neurons following ischemic injury, sigma receptor-mediated neuroprotection, such as that reported by our laboratory (Ajmo et al., 2006), is likely due in part to the preservation of intracellular calcium homeostasis in these cells. Thus, sigma receptors are a viable target for neuroprotection following ischemia and possibly other neurodegenerative diseases involving excitotoxicity.

ACKNOWLEDGMENTS

We thank Nivia Cuevas, RPh, for comments on a draft of this manuscript.

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FOOTNOTES

*Grant support for this work was provided by AHA Florida/Puerto Rico Affiliate Award Grant-In-Aid to J.C.

A preliminary report of some of these results has been presented in abstract form [Zhang, H., Guerrero, W., DeMesquita, D., Pennypacker, K., Cuevas, J. (2004). *Soc. Neurosci. Abst.* 1019.1].

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LEGENDS FOR FIGURES

Figure 1. Increases $[Ca^{2+}]_i$ evoked by ischemia are dependent on the number of days the neurons have been in culture. A, Change in $[Ca^{2+}]_i$ ($[Ca^{2+}]_i$ minus baseline $[Ca^{2+}]_i$) as a function of time for two neurons collected from the same neuronal isolation. Neurons were in culture for 3 (gray line) or 14 (black line) days. Line above traces indicates application of chemical ischemia (0 glucose, 4 mM azide). B, Bar graph of mean change in $[Ca^{2+}]_i$ ($\Delta[Ca^{2+}]_i$) obtained in response to chemical ischemia when neurons were held in culture for the indicated time points ($n = 8-34$). Asterisks denote significant difference from 3 days ($p < 0.05$) and dagger indicates significant difference from 21 days ($p < 0.05$). C, Change in $[Ca^{2+}]_i$ as a function of time for a single neuron exposed to chemical ischemia for 2 min (line above trace) in the absence (Control) and presence of 200 nM TTX (TTX). D, Bar graph of mean change in $[Ca^{2+}]_i$ obtained in response to chemical ischemia in the absence (Control) and presence of 200 nM TTX (TTX) ($n = 49$). Asterisks denote significant difference from Control ($p < 0.001$).

Figure 2. Increases $[Ca^{2+}]_i$ evoked by ischemia are dependent on both extracellular calcium and activation of IP_3 -sensitive stores. A, Bar graph of intracellular calcium levels recorded in the absence (0 Ca, $n = 69$) and presence (Control, $n = 147$) of external calcium prior to induction of ischemia (Baseline) and for peak response under these conditions during the ischemic insult (Peak). Asterisks denote significant difference from respective baselines ($p < 0.05$), and pound symbol indicates significant difference from peak control ($p < 0.05$). B, Bar graph of mean change in $[Ca^{2+}]_i$ ($\Delta[Ca^{2+}]_i$) obtained in response to chemical ischemia in the absence (0 Ca) and presence (Control) of extracellular calcium. Asterisks indicate significant difference from control ($p < 0.001$). Bar graph of mean baseline and peak $[Ca^{2+}]_i$ (C) and $\Delta[Ca^{2+}]_i$ (D) obtained in response to ischemia in the absence (Control; $n = 46$) and presence of 100 μ M D-AP5 (AP5, $n =$

87) and 10 μM La^{3+} ($n = 123$). Asterisks denotes significant difference from respective baselines (C) and Control (D), pound symbol indicates significant difference from peak control (C) and AP5 (D), and dagger indicates significant difference from AP5 (C); $p < 0.05$ in all cases. Bar graph of mean baseline and peak $[\text{Ca}^{2+}]_i$ (E) and $\Delta[\text{Ca}^{2+}]_i$ (F) evoked by chemical ischemia in the absence (Control; $n = 292$) and presence of ryanodine (20 μM ; $n = 105$), ruthenium red (100 μM , $n = 109$), or following preincubation in thapsigargin (10 μM , 45 min, 23° C; $n = 194$). Asterisks indicate significant difference from respective baselines, and pound symbols indicate significant difference from peak $[\text{Ca}^{2+}]_i$ (E) or $\Delta[\text{Ca}^{2+}]_i$ (F) under control conditions and in the presence of ryanodine ($p < 0.05$).

Figure 3. The sigma receptor-selective ligand, DTG, blocks elevations in $[\text{Ca}^{2+}]_i$ evoked by in vitro ischemia. A, $[\text{Ca}^{2+}]_i$ as a function of time recorded from a single neuron during ischemic insults in the absence (Control) and presence of 50 μM DTG. B, Mean peak $[\text{Ca}^{2+}]_i$ measured during an ischemic episode in the absence and presence of 50 μM DTG ($n = 13$). Asterisks indicate significant difference from Control ($p < 0.001$). C, Bar graph of mean relative $\Delta[\text{Ca}^{2+}]_i$ ($\Delta[\text{Ca}^{2+}]_i/\text{mean } \Delta[\text{Ca}^{2+}]_i$ for control in the absence of DTG) obtained in response to chemical ischemia in the absence (Control; $n = 758$) and presence of ruthenium red (100 μM , RR, $n = 109$), D-AP5 (100 μM , AP5, $n = 87$), La^{3+} (10 μM , $n = 123$), nominal extracellular Ca^{2+} (0 Ca, $n = 69$), and TTX (200 nM, $n = 279$) or following preincubation in thapsigargin (10 μM , THAP, 45 min, 23° C, $n = 194$) without DTG (Drug); or when 100 μM DTG was present (Drug + DTG; Control, $n = 111$; RR, $n = 135$; AP5, $n = 135$; La^{3+} , $n = 136$; 0 Ca, $n = 103$; TTX, $n = 87$; THAP, $n = 211$). Asterisks indicate significant difference between DTG and DTG-free conditions for each test group.

Figure 4. Inhibition of sigma receptors by metaphit blocks DTG-mediated suppression of ischemia-evoked increases in $[Ca^{2+}]_i$. A, $[Ca^{2+}]_i$ as a function of time recorded from 4 different neurons during ischemic insults in the absence (Control) and presence of 50 μ M DTG (DTG) and following preincubation in 50 μ M metaphit (MET) or in the presence of DTG following metaphit preincubation (MET+DTG). B, Mean peak $[Ca^{2+}]_i$ measured during ischemic episodes under the indicated conditions (n = 19-29). Asterisks, pound symbol, and dagger denote significant difference from Control, DTG alone (DTG), and metaphit alone (MET) groups, respectively (p < 0.05). C, Change in $[Ca^{2+}]_i$ recorded in the presence of DTG in control cells (DTG; n = 13) and cells preincubated in metaphit (MET + DTG; n = 27) normalized to the mean $\Delta[Ca^{2+}]_i$ recorded for the control of each test group (eg. Control and MET). Asterisks indicate significant difference (p < 0.001).

Figure 5. Concentration-dependent relief of DTG inhibition of ischemia-induced transient elevations in cytoplasmic Ca^{2+} by the sigma receptor antagonist, BD-1047. A, typical traces of $[Ca^{2+}]_i$ recorded from three E18 neurons in response to ischemia under control conditions (solid black line), with 100 μ M DTG (gray solid grey line) or with 100 μ M DTG and 10 μ M BD-1047 (dotted black line). B, Bar graph of relative changes in $[Ca^{2+}]_i$ for neurons exposed to ischemia under control conditions (Control, n=77 cells), with 100 μ M DTG (n=83 cells), with 100 μ M DTG and 1 μ M BD-1047 (DTG +1 μ M BD, n=93 cells) and with 100 μ M DTG plus 10 μ M BD-1047 (DTG + 10 μ M BD, n=145 cells). The asterisks denote significant difference (p < 0.05) from control and pound symbols denote significant difference (p < 0.05) from 100 μ M DTG treatment.

Figure 6. Activation of sigma 1 receptors in cortical neurons depresses ischemia-induced elevations in $[Ca^{2+}]_i$. A, $[Ca^{2+}]_i$ as a function of time recorded from three different neurons during

an ischemic insult in the absence (Control) and presence of carbetapentane (CBP) at the indicated concentrations. B, Mean peak $[Ca^{2+}]_i$ measured during ischemic episodes with the indicated concentrations of carbetapentane (n = 57-107). Solid line is a best fit to the data using a single-site Langmuir-Hill equation with an IC_{50} value of 13.3 μ M and a Hill coefficient of 0.8.

Figure 7. The sigma-2 receptor agonist, ibogaine, fails to block ischemia-induced elevations in $[Ca^{2+}]_i$ in cortical neurons. A. Representative traces of $[Ca^{2+}]_i$ recorded from two neurons in the absence (Control) and presence of 100 μ M ibogaine (IBO). B. Mean peak $[Ca^{2+}]_i$ measured in cortical neurons during ischemic episodes in the absence (Control) and presence of ibogaine at the indicated concentrations (n= 4-9).

Figure 8. Concentration-dependent reduction of ischemia induced transient elevations in cytoplasmic Ca^{2+} by the sigma-1 receptor agonists (+)-pentazocine and PRE-084. A, typical traces of $[Ca^{2+}]_i$ recorded from E18 neurons in response to either ischemia (solid black line), or ischemia in the presence of (+)-pentazocine (PTZ; 10 μ M, gray solid grey line; 100 μ M, dashed black line). B, bar graph of relative changes in $[Ca^{2+}]_i$ for neurons exposed to ischemia (Control, n=104), ischemia and 10 μ M (+)-pentazocine (10 μ M PTZ, n=90) and 100 μ M (+)-pentazocine (100 μ M PTZ, n=104). The asterisks denote significant difference ($p < 0.05$) from control. C, Representative traces of $[Ca^{2+}]_i$ recorded from three E18 neurons in response to either ischemia under control conditions (solid black line), with 10 μ M PRE-084 (gray solid grey line) or with 100 μ M PRE-084 (dotted black line). D, bar graph of relative changes in $[Ca^{2+}]_i$ for neurons exposed to ischemia in the absence (Control, n=432 cells), and presence of 10 μ M PRE-084 (10 μ M PRE, n=110 cells) and 100 μ M PRE-084 (100 μ M PRE, n=122 cells). The asterisks denote significant difference ($p < 0.05$) from control and the pound symbols denote significant difference ($p < 0.05$) from azide and 10 μ M PRE-084 treatment.

Figure 9. Spontaneous Ca^{2+} transients are blocked by sigma receptor agonists. A, typical traces of $[\text{Ca}^{2+}]_i$ recorded from a single neuron prior to (Control), during and following washout (Wash) of bath applied 100 μM DTG (*i.*), 50 μM ibogaine (*ii.*, IBO), and 100 μM carbetapentane (*iii.*, CBP). Bar graph of mean frequency of events detected under conditions in (A) using the sigma ligands DTG (B, $n = 59$), ibogaine (C, $n = 77$), and carbetapentane (D, $n = 21$). Asterisks denote significant difference ($p < 0.05$) from respective controls.

Figure 1

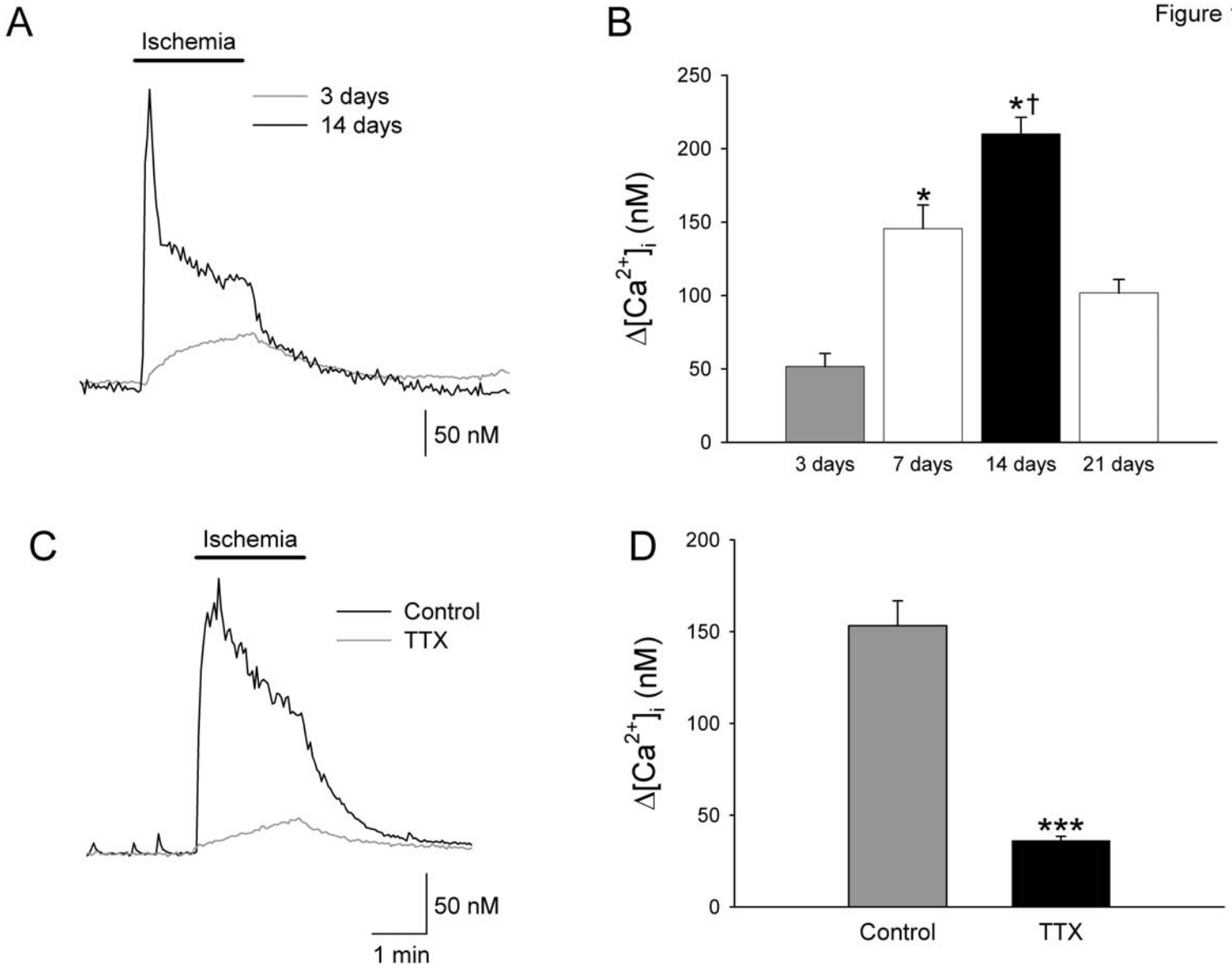
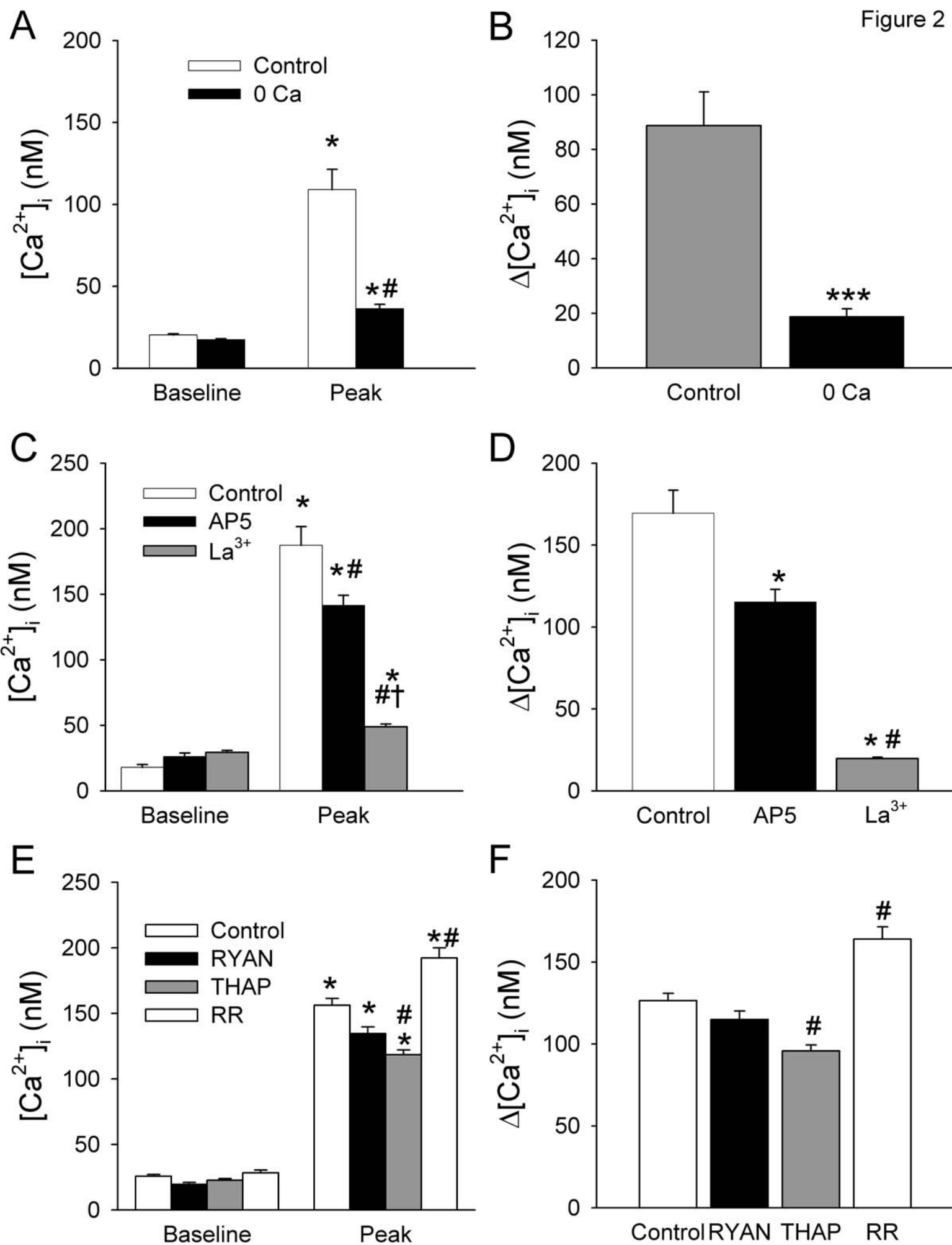


Figure 2



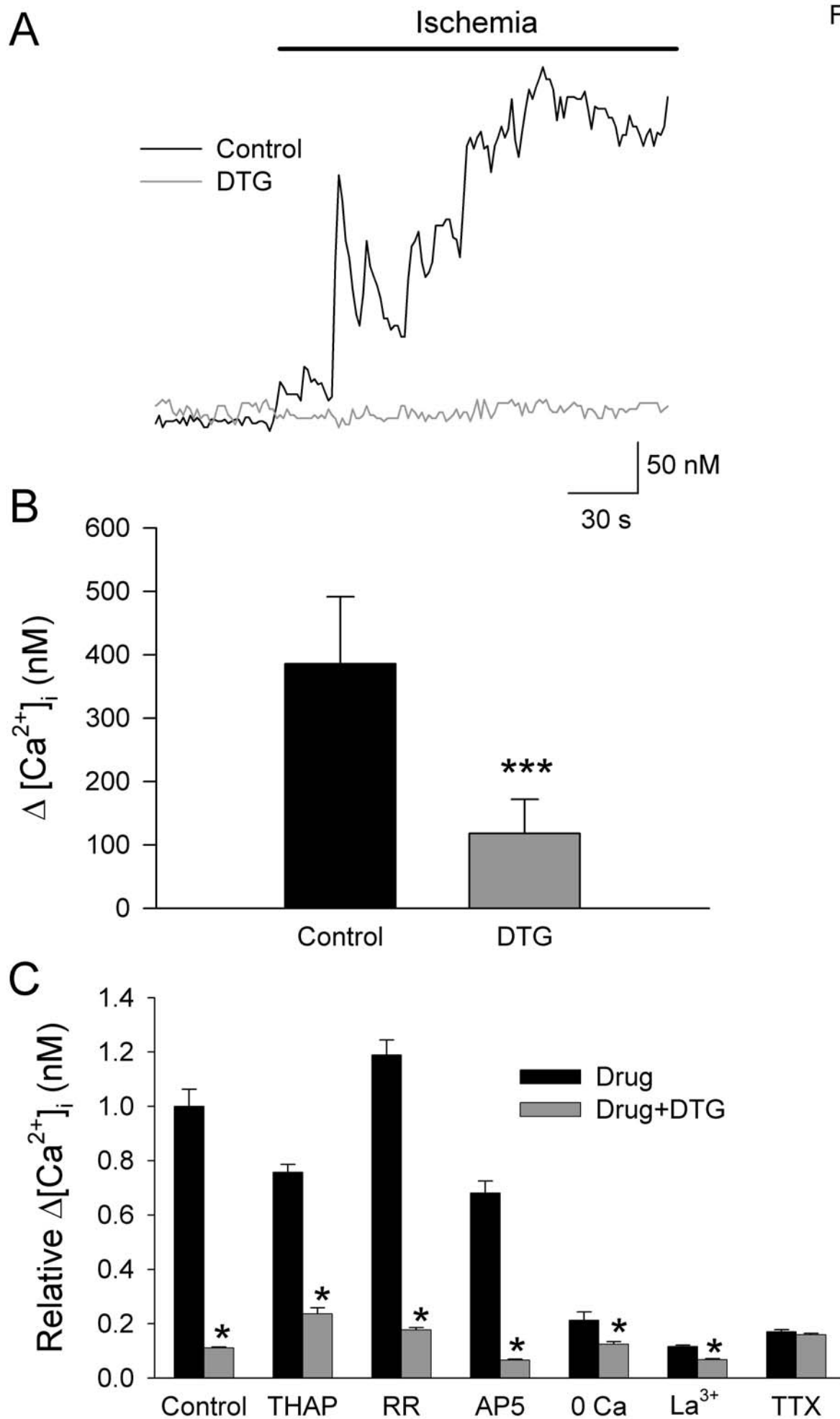
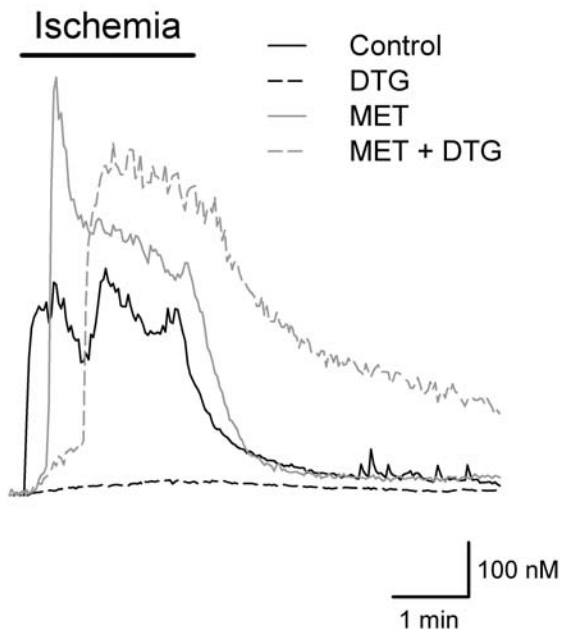
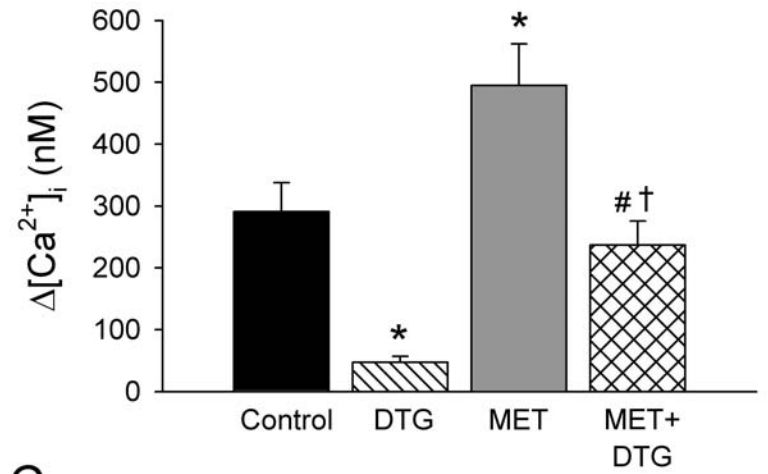


Figure 4

A



B



C

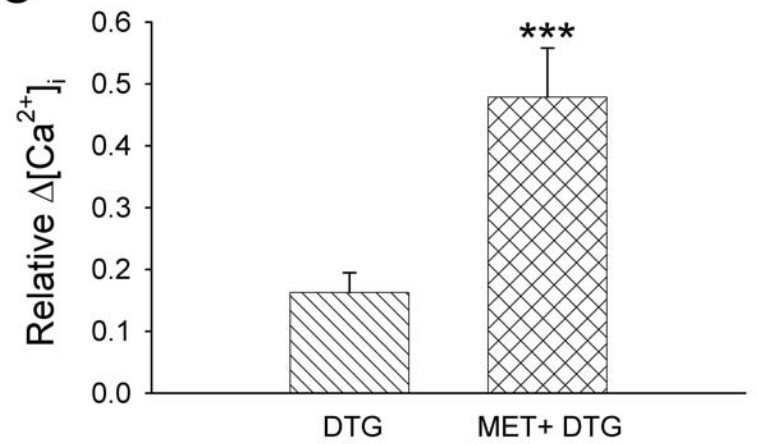


Figure 5

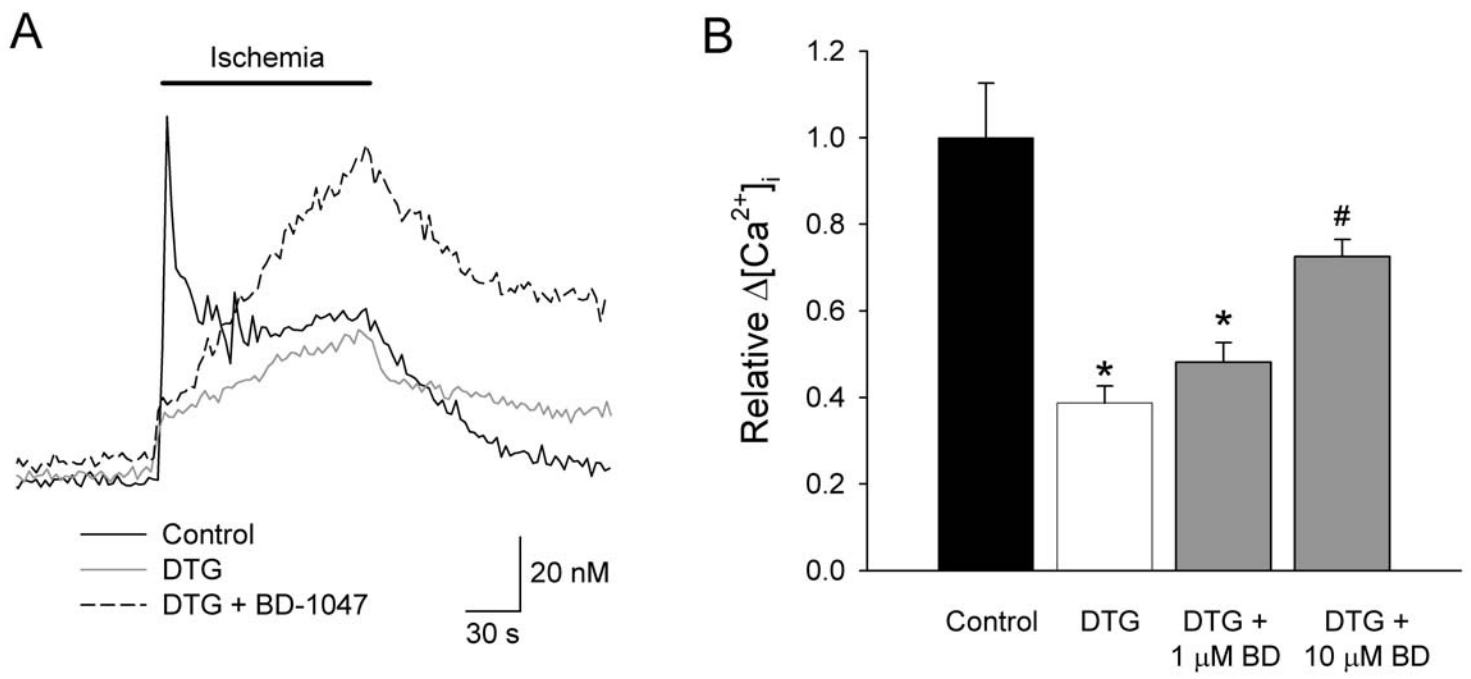


Figure 6

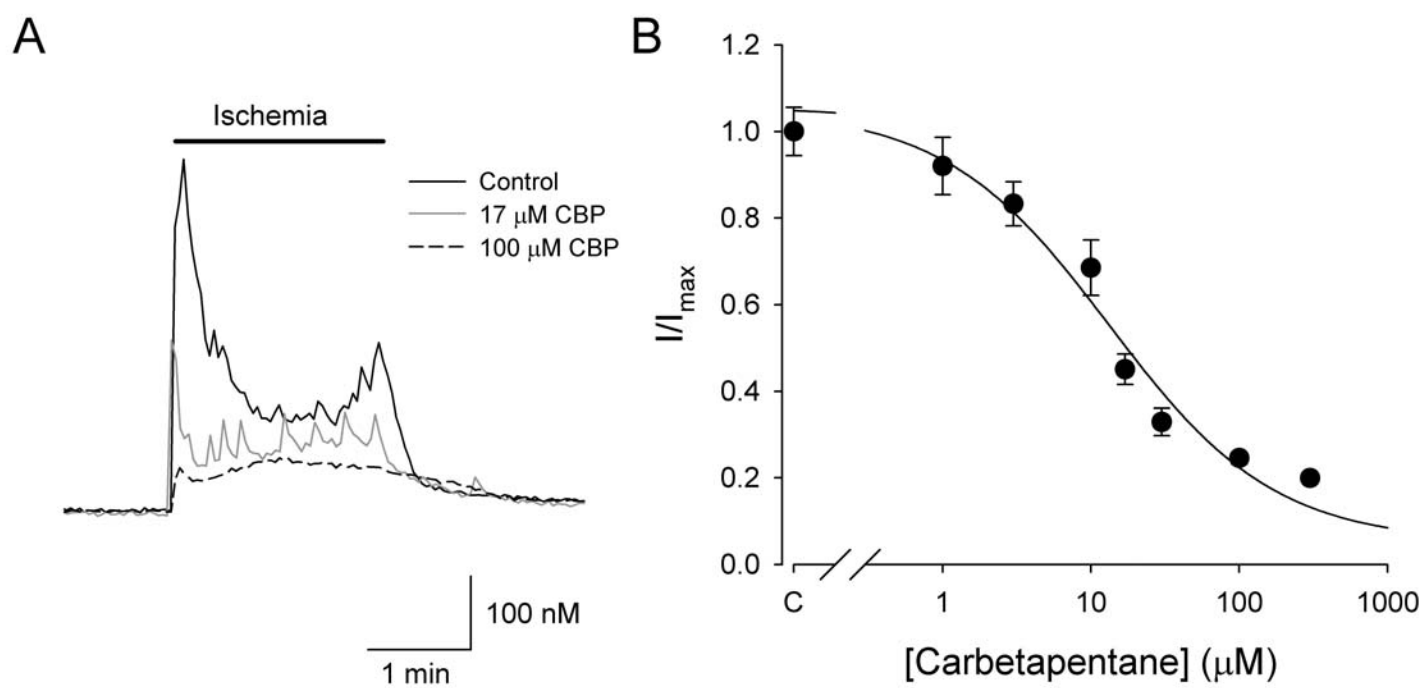


Figure 7

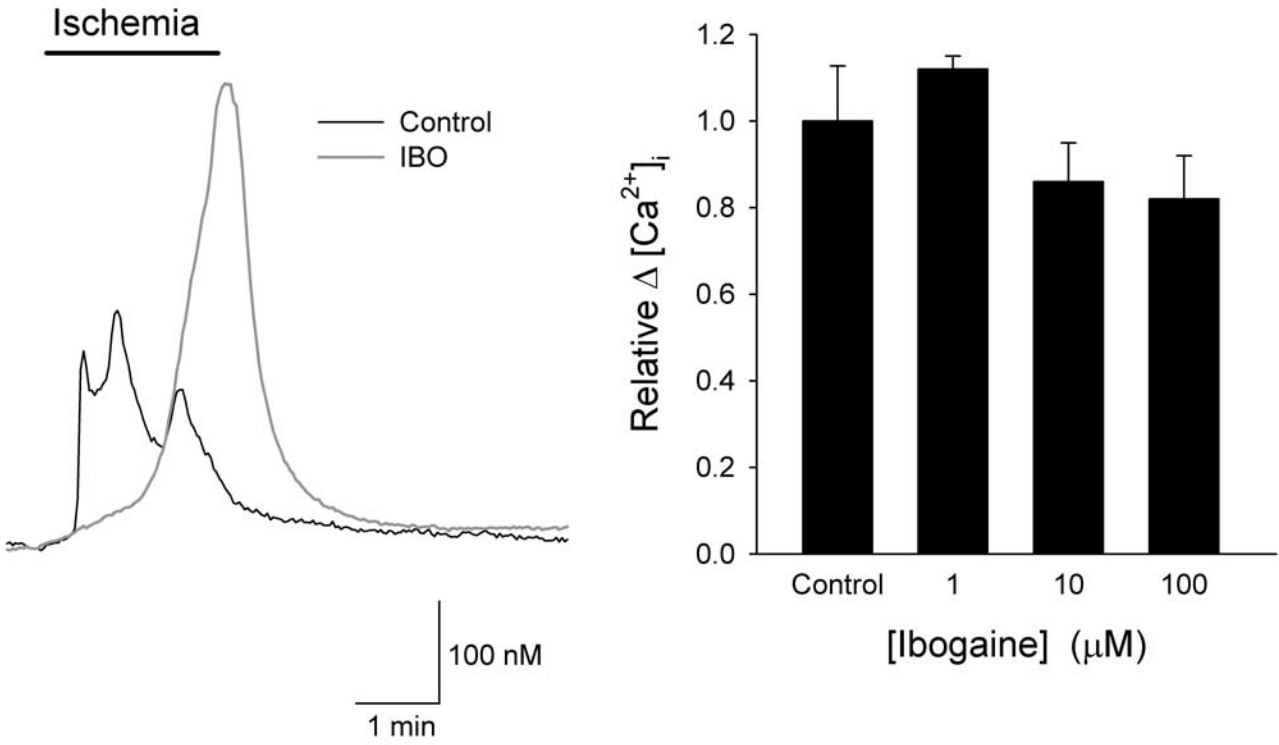


Figure 8

